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(54) Title: GHRELIN ANALOGS

(57) Abstract: The present invention features truncated ghrelin analogs active at the GHS receptor. Ghrelin is a naturally occurring modified peptide. The analogs can bind to the GHS receptor and, preferably, bring about signal transduction. Ghrelin analogs have a variety of different uses including being used as a research tool and being used therapeutically.



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TITLE OF THE INVENTION
GHRELIN ANALOGS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 The present application claims priority to provisional application U.S. Serial No. 60/207,920, filed May 30, 2000, hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

10 The references cited herein are not admitted to be prior art to the claimed invention.

 The pulsatile release of growth hormone from the pituitary somatotrops is regulated by two hypothalamic neuropeptides: growth hormone-releasing hormone and somatostatin. Growth hormone-releasing hormone stimulates release of growth hormone, whereas, somatostatin inhibits secretion of growth hormone. (Frohman *et al.*, *Endocrinol. Rev.* 1986, 7, 223-253, and Strobl *et al.*, *Pharmacol. Rev.* 1994, 46, 1-34.)

 Release of growth hormone from the pituitary somatotrops can also be controlled by growth hormone-releasing peptides. A hexapeptide His-D-Trp-Ala-Trp-D-Phe-Lys-amide (GHRP-6), was found to release growth hormone from somatotrops in a dose-dependent manner in several species including man. (Bowers *et al.*, *Endocrinology* 1984, 114, 1537-1545.) Subsequent chemical studies on GHRP-6 led to the identification of other potent growth-hormone secretagogues such as GHRP-1, GHRP-2 and hexarelin (Cheng *et al.*, *Endocrinology* 1989, 124, 2791-2798, Bowers, C. Y. Novel GH-Releasing Peptides. In: *Molecular and Clinical Advances in Pituitary Disorders*. Ed: Melmed, S.; Endocrine Research and Education, Inc., Los Angeles, CA, USA 1993, 153-157, and Deghenghi *et al.*, *Life Sci.* 1994, 54, 1321-1328):

GHRP-1 Ala-His-D-(2')-Nal-Ala-Trp-D-Phe-Lys-NH₂

GHRP-2 D-Ala-D-(2')-Nal-Ala-Trp-D-Nal-Lys-NH₂

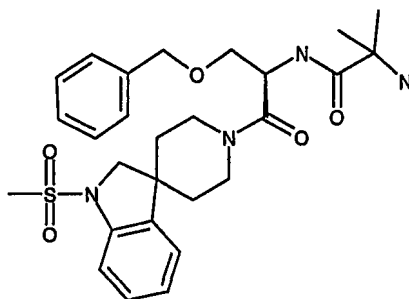
Hexarelin His-D-2-MeTrp-Ala-Trp-D-Phe-Lys-NH₂

30 GHRP-1, GHRP-2, GHRP-6, and hexarelin are synthetic growth-hormone secretagogues. Growth-hormone secretagogues stimulate secretion of growth hormone by a mechanism different from that of growth hormone-releasing hormone, but like growth hormone-releasing hormone, they antagonize release of somatostatin from the pituitary and hypothalamus. (Bowers *et al.*, *Endocrinology* 1984, 114, 1537-1545, Cheng *et al.*, *Endocrinology* 1989, 124, 2791-2798, Bowers,

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C. Y. Novel GH-Releasing Peptides. In: *Molecular and Clinical Advances in Pituitary Disorders*. Ed: Melmed, S.; Endocrine Research and Education, Inc., Los Angeles, CA, USA 1993, 153-157, and Deghenghi *et al.*, *Life Sci.* 1994, 54, 1321-1328.)

- The low oral bioavailability (< 1%) of the peptidyl growth-hormone secretagogues stimulated search for non-peptide compounds mimicking action of GHRP-6 in the pituitary. Several benzolactams and spiroindanes have been reported to stimulate growth-hormone release in various animal species and in man. (Smith *et al.*, *Science* 1993, 260, 1640-1643, Patchett *et al.*, *Proc. Natl. Acad. Sci. USA* 1995, 92, 7001-7005, and Chen *et al.*, *Bioorg. Med. Chem. Lett.* 1996, 6, 2163-2169.) A specific example of a small spiroindane is MK-0677 (Patchett *et al. Proc. Natl. Acad. Sci. USA* 1995, 92, 7001-7005):



- The actions of the above-mentioned growth-hormone secretagogues (both peptide and non-peptide) appear to be mediated by a specific growth-hormone secretagogue receptor (GHS receptor). (Howard *et al.*, *Science* 1996, 273, 974-977, and Pong *et al.*, *Molecular Endocrinology* 1996, 10, 57-61.) This receptor is present in the pituitary and hypothalamus of various mammalian species (GHSR1a) and is distinct from the growth hormone-releasing hormone receptor. The GHS receptor was also detected in the other areas of the central nervous system and in peripheral tissues, for instance adrenal and thyroid glands, heart, lung, kidney and skeletal muscles. (Chen *et al.*, *Bioorg. Med. Chem. Lett.* 1996, 6, 2163-2169, Howard *et al.*, *Science* 1996, 273, 974-977, Pong *et al.*, *Molecular Endocrinology* 1996, 10, 57-61, Guan *et al.*, *Mol. Brain Res.* 1997, 48, 23-29, and McKee *et al.*, *Genomics* 1997, 46, 426-434.) A truncated version of GHSR1a has been reported. (Howard *et al.*, *Science* 1996, 273, 974-977.)

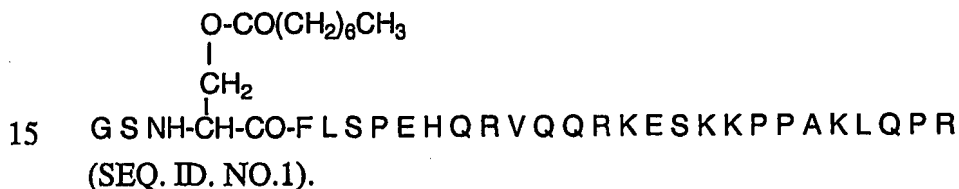
- The GHS receptor is a G-protein coupled-receptor. Effects of GHS receptor activation includes depolarization and inhibition of potassium channels, an increase in intercellular concentrations of inositol triphosphate (IP3), and a transient

increase in the concentrations of intracellular calcium. (Pong *et al.*, *Molecular Endocrinology* 1996, 10, 57-61, Guan *et al.*, *Mol. Brain Res.* 1997, 48, 23-29, and McKee *et al.*, *Genomics* 1997, 46, 426-434.)

5 SUMMARY OF THE INVENTION

The present invention features truncated ghrelin analogs active at the GHS receptor. Ghrelin is a naturally occurring modified peptide. The analogs can bind to the GHS receptor and, preferably, bring about signal transduction. Ghrelin analogs have a variety of different uses including being used as a research tool and
10 being used therapeutically.

The structure of human ghrelin is as follows (where the chemical structure of a modified serine is shown, and unmodified amino acids are noted using one letter codes):



A core region present in ghrelin was found to provide for activity at the GHS receptor. The core region comprises the four N-terminal amino acids, where the
20 serine at position 2 or 3 is modified with a bulky hydrophobic R group.

Thus, a first aspect of the present invention describes a truncated ghrelin analog having a structure selected from the group consisting of:

- a) $\text{Z}^1\text{-GSXF(Z)}_n\text{-Z}^2$; and
- b) $\text{Z}^1\text{-GXSF(Z)}_n\text{-Z}^2$;

25 wherein X is a modified amino acid containing a bulky hydrophobic R group;

each Z is independently either alanine, valine, leucine, isoleucine, proline, tryptophan, phenylalanine, methionine, glycine, serine, threonine, tyrosine, cysteine, asparagine, glutamine, lysine, arginine, histidine, aspartic acid, or glutamic
30 acid, or a derivative thereof;

Z^1 is an optionally present protecting group that, if present, is covalently joined to the N-terminal amino group;

Z² is an optionally present protecting group that, if present, is covalently joined to the C-terminal carboxy group; and

n is 0 to 19;

or a pharmaceutically acceptable salt thereof.

5 Unless otherwise stated, those amino acids with a chiral center are provided in the L-enantiomer. Reference to "a derivative thereof" refers to a modified amino acid such as the corresponding D-amino acid, a N-alkyl-amino acid, a β -amino acid, or a labeled amino acid.

10 Another aspect of the present invention describes a method of screening for a compound able to bind to a GHS receptor. The method comprises the step of measuring the ability of a compound to affect binding of a truncated ghrelin analog to either the receptor, a fragment of the receptor comprising a ghrelin binding site, a polypeptide comprising the fragment, or a derivative of the polypeptide.

15 Another aspect of the present invention describes a method for achieving a beneficial affect in a subject comprising the step of administering to the subject an effective amount of a ghrelin analog. The effective amount produces a beneficial effect in helping to treat (e.g., cure or reduce the severity) or prevent (e.g., reduce the likelihood of onset or severity) a disease or disorder.

20 Another aspect of the present invention describes a method for stimulating growth hormone secretion comprising the step of administering to a subject an effective amount of a ghrelin analog. The effective amount is at least an amount sufficient to produce a detectable increase in growth hormone secretion and, preferably, is an amount sufficient to achieve a beneficial affect in a patient.

25 Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention features truncated ghrelin analogs active at the GHS receptor. Human ghrelin is a 28 amino acid modified peptide where a serine hydroxyl group is esterified by n-octanoic acid. (Kojima *et al.*, *Nature* 1999, 402,

656-660, and Kojima, (Abstract), Third International Symposium on Growth Hormone Secretagogues, Keystone, Colorado, USA 2000, February 17-19.)

Ghrelin induces growth hormone release from primary-culture pituitary cells in a dose-dependent manner without stimulating the release of the other pituitary hormones. Injected intravenously into anaesthetized rats, ghrelin stimulated pulsatile release of growth hormone. (Kojima *et al.*, *Nature* 1999, 402, 656-660.)

Truncated ghrelin analogs described herein are active at the GHS receptor. The analogs can bind to the receptor, and preferably, stimulate receptor activity. Ghrelin analogs have a variety of different uses including being used as a research tool and being used therapeutically.

Research tool applications generally involve the use of a truncated ghrelin analog and the presence of a GHS receptor or fragment thereof. The GHS receptor can be present in different environments such as a mammalian subject, a whole cell, or membrane fragments. Examples of research tool applications include screening for compounds active at the GHS receptor, determining the presence of the GHS receptor in a sample or preparation, and examining the role or effect of ghrelin.

Ghrelin analogs can be used to screen for both ghrelin agonists and ghrelin antagonists. Screening for ghrelin agonists can be performed, for example, by using a ghrelin analog in a competition experiment with test compounds. Screening for ghrelin antagonists can be performed, for example, by using a ghrelin analog to produce GHS receptor activity and then measuring the ability of a compound to alter GHS receptor activity.

Ghrelin analogs can be administered to a subject. A "subject" refers to a mammal including, for example, a human, a rat, a mouse, or a farm animal. Reference to subject does not necessarily indicate the presence of a disease or disorder. The term subject includes, for example, mammals being dosed with a truncated ghrelin analog as part of an experiment, mammals being treated to help alleviate a disease or disorder, and mammals being treated prophylactically to retard or prevent the onset of a disease or disorder.

Ghrelin agonists can be used to achieve a beneficial effect in a subject such as one or more of the following: treating a growth hormone deficient state, increasing muscle mass, increasing bone density, treating sexual dysfunction in males or females, facilitating a weight gain, facilitating maintenance of weight, facilitating maintenance of physical functioning, facilitating recovery of physical function, and/or facilitating appetite increase. Facilitating a weight gain, maintenance in weight, or

appetite increase is particularly useful for a patient having a disease or disorder, or under going a treatment, accompanied by weight loss. Examples of diseases or disorders accompanied by weight loss include anorexia, bulimia, cancer cachexia, AIDS, wasting, cachexia, and wasting in frail elderly. Examples of treatments
5 accompanied by weight loss include chemotherapy, radiation therapy, temporary or permanent immobilization, and dialysis.

Ghrelin antagonists can also be used to achieve a beneficial effect in a patient. For example, a ghrelin antagonist can be used to facilitate weight loss, facilitate appetite decrease, facilitate weight maintenance, treat obesity, treat diabetes,
10 treat complications of diabetes including retinopathy, and/or treat cardiovascular disorders. Excessive weight is a contributing factor to different diseases including hypertension, diabetes, dyslipidemias, cardiovascular disease, gall stones, osteoarthritis and certain forms of cancers. Bringing about a weight loss can be used, for example, to reduce the likelihood of such diseases and as part of a treatment for
15 such diseases.

Truncated Ghrelin Analogs

The smaller size of truncated ghrelin analogs offers advantages over longer-length ghrelin such as ease of synthesis and/or increased solubility in
20 physiological buffers. In addition, small analogs can serve as models for producing peptidomimetics having desirable pharmacological properties.

Truncated ghrelin analogs described herein have the structure:

$Z^1\text{-GSXF(Z)}_n\text{-Z}^2$ or $Z^1\text{-GXSF(Z)}_n\text{-Z}^2$; preferably, $Z^1\text{-GSXF(Z)}_n\text{-Z}^2$.
25

wherein X is a modified amino acid containing a bulky hydrophobic R group;

each Z is independently either alanine, valine, leucine, isoleucine, proline, tryptophan, phenylalanine, methionine, glycine, serine, threonine, tyrosine,
30 cysteine, asparagine, glutamine, lysine, arginine, histidine, aspartic acid, or glutamic acid, or a derivative thereof;

Z^1 is an optionally present protecting group that, if present, is covalently joined to the N-terminal amino group;

Z^2 is an optionally present protecting group that, if present, is
35 covalently joined to the C-terminal carboxy group; and

n is 0 to 19; in different embodiments n is 0-3, 0-6, 0-11, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11;

or a pharmaceutically acceptable salt thereof.

Individual amino acids such as those present in the general structure of
5 a truncated ghrelin analog or a $(Z)_n$ group can be represented as follows:

A=Ala=Alanine; C=Cys=Cysteine; D=Asp=Aspartic acid; E=Glu=Glutamic acid;

F=Phe=Phenylalanine; G=Gly=Glycine; H=His=Histidine; I=Ile=Isoleucine;

K=Lys=Lysine; L=Leu=Leucine; M=Met=Methionine; N=Asn=Asparagine;

P=Pro=Proline; Q=Gln=Glutamine; R=Arg=Arginine; S=Ser=Serine;

10 T=Thr=Threonine; V=Val=Valine; W=Trp=Tryptophan; and Y=Tyr=Tyrosine.

The present invention includes diastereomers as well as their racemic and resolved enantiomerically pure forms. Truncated ghrelin analogs can contain D-amino acids, L-amino acids or a combination thereof. Preferably, amino acids present in a truncated ghrelin analog are the L-enantiomer.

15 Since each Z is independently selected, the resulting $(Z)_n$ group is not limited to a group of contiguous amino acids present in ghrelin. In an embodiment of the present invention $(Z)_n$ is a contiguous amino acid group present in ghrelin.

The design of analogs where $(Z)_n$ is a contiguous amino acid group not present in ghrelin can be based on substitutions to ghrelin, or a truncated ghrelin,
20 where the substitutions take into account differences in amino acids R groups. An R group affects different properties of the amino acid such as physical size, charge, and hydrophobicity. Amino acids can be divided into different groups as follows: neutral and hydrophobic (alanine, valine, leucine, isoleucine, proline, tryptophan, phenylalanine, and methionine); neutral and polar (glycine, serine, threonine, tyrosine,
25 cysteine, asparagine, and glutamine); basic (lysine, arginine, and histidine); and acidic (aspartic acid and glutamic acid).

Generally, in substituting different amino acids it is preferable to exchange amino acids having similar properties. Substituting different amino acids within a particular group, such as substituting valine for leucine, arginine for lysine,
30 and asparagine for glutamine are good candidates for not causing a change in peptide functioning.

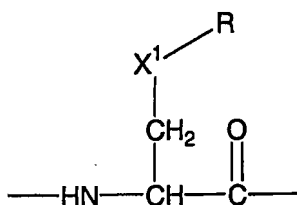
Preferred derivatives are D-amino acids, N-alkyl-amino acids, β -amino acids, or a labeled amino acid (including a labeled version of a D-amino acid, a N-alkyl-amino acids, or a β -amino acid). A labeled derivative indicates the alteration of
35 an amino acid or amino acid derivative with a detectable label. Examples of

detectable labels include luminescent, enzymatic, and radioactive labels. Both the type of label and the position of the label can effect analog activity. Labels should be selected and positioned so as not to substantially alter the activity of the truncated ghrelin analog at the GHS receptor. The effect of a particular label and position on ghrelin activity can be determined using assays measuring ghrelin activity and/or binding.

A protecting group covalently joined to the N-terminal amino group reduces the reactivity of the amino terminus under *in vivo* conditions. Amino protecting groups include -C₁₋₁₀ alkyl, -C₁₋₁₀ substituted alkyl, -C₂₋₁₀ alkenyl, -C₂₋₁₀ substituted alkenyl, aryl, -C₁₋₆ alkyl aryl, -C(O)-(CH₂)₁₋₆-COOH, -C(O)-C₁₋₆ alkyl, -C(O)-aryl, -C(O)-O-C₁₋₆ alkyl, or -C(O)-O-aryl. Preferably, the amino terminus protecting group is acetyl, propyl, succinyl, benzyl, benzyloxycarbonyl or *t*-butyloxycarbonyl.

A protecting group covalently joined to the C-terminal carboxy group reduces the reactivity of the carboxy terminus under *in vivo* conditions. The carboxy terminus protecting group is preferably attached to the α -carbonyl group of the last amino acid. Carboxy terminus protecting groups include amide, methylamide, and ethylamide.

In a preferred embodiment X has the structure:



wherein X¹ is either -O-, -S-, -OC(O)-, -NHC(O)-, or -CH₂-; preferably, X¹ is either -OC(O)- or -NHC(O)-, more preferably, X¹ is -OC(O)-; and R is either a longer length alkyl, substituted longer length alkyl, longer length alkenyl, substituted longer length alkenyl, longer length heteroalkyl, substituted longer length heteroalkyl, aryl, or alkylaryl; preferably, R is either -C₄₋₂₀ alkyl, -C₄₋₂₀ substituted alkyl, -C₄₋₂₀ substituted alkenyl, -C₄₋₂₀ alkenyl, -C₄₋₂₀ heteroalkyl, -C₄₋₂₀ substituted heteroalkyl, aryl, or alkylaryl; more preferably, R is a -C₅₋₁₅ alkyl or a -C₅₋₁₅ substituted alkyl; more preferably R is a -C₅₋₁₅; more preferably, R is -(CH₂)₆CH₃.

Examples of truncated ghrelin analogs including the following modified peptides:

GSXFLSPEHQRVQQ (compound 13, SEQ. ID. NO. 2),

GSX^bFLSPEHQRVQQ (compound 14, SEQ. ID. NO. 3),

5 GSXFLSPEHQRVQQRKESKKPPA-NH₂ (compound 18, SEQ. ID. NO. 4),

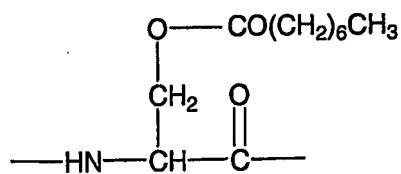
GSXFLSPEHQRVQQRKES-NH₂ (compound 19, SEQ. ID. NO. 5),

GSXFLSPEHQ-NH₂ (compound 20, SEQ. ID. NO. 6),

GSXFL-NH₂ (compound 21),

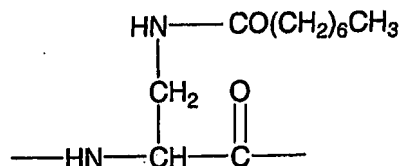
GSXF-NH₂ (compound 22),

10 wherein X is



and X^b is

15



The effect of particular groups, such as X, (Z)_n or blocking groups on ghrelin analog activity at the GHS receptor can be determined using techniques such as those described in the examples provided below. In different embodiments a truncated ghrelin analog has at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90%, functional activity relative to ghrelin as determined using the assay described in the Example 3 *infra*; and/or has an IC₅₀ greater than about 1,000, greater than about 100, or greater than about 50 using the binding assay described in Example 2 *infra*. With respect to IC₅₀, greater refers to potency and thus indicates a lesser amount is needed to achieve binding inhibition.

Truncated ghrelin analogs can be produced using techniques well known in the art. For example, a polypeptide region of a truncated ghrelin analog can be chemically or biochemically synthesized and modified. Techniques for chemical synthesis of polypeptides are well known in the art. (See *e.g.*, Vincent in *Peptide and*

- Protein Drug Delivery*, New York, N.Y., Dekker, 1990.) Examples of techniques for biochemical synthesis involving the introduction of a nucleic acid into a cell and expression of nucleic acids are provided in Ausubel, *Current Protocols in Molecular Biology*, John Wiley, 1987-1998, and Sambrook *et al.*, in *Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989.

Chemical Definitions

Definitions of some of the chemical groups making up a protecting group or present in an R group are provided below.

- 10 “Alkyl” refers to a hydrocarbon group containing one or more carbon atoms, where multiple carbon atoms if present are joined by single bonds. The alkyl hydrocarbon group may be straight-chain or contain one or more branches or cyclic groups. Protecting group hydrocarbons are preferably C₁₋₄. R alkyl groups are preferably about 4 to about 20 carbon atoms in length, more preferably C₅₋₁₅, and
15 more preferably -(CH₂)₆CH₃. Reference to “longer length alkyl” indicates at least about 4 carbon atoms.

- “Substituted alkyl” refers to an alkyl wherein one or more hydrogens of the hydrocarbon group are replaced with one or more substituents selected from the group consisting of halogen (preferably -F or -Cl), -OH, -CN, -SH, -NH₂, -NHCH₃,
20 -NO₂, -C₁₋₂ alkyl substituted with 1 to 6 halogens (preferably -F or -Cl, more preferably -F), -CF₃, -OCH₃, -OCF₃, and -(CH₂)₀₋₄-COOH. In different embodiments 1, 2, 3 or 4 substituents are present. The presence of -(CH₂)₀₋₄-COOH results in the production of an alkyl acid. Examples of alkyl acids containing, or consisting of, -(CH₂)₀₋₄-COOH include 2-norbornane acetic acid, tert-butyric acid and 3-cyclopentyl
25 propionic acid.

 “Heteroalkyl” refers to an alkyl wherein one of more of the carbon atoms in the hydrocarbon group are replaced with one or more of the following groups: amino, amido, -O-, or carbonyl. In different embodiments 1 or 2 heteroatoms are present.

- 30 “Substituted heteroalkyl” refers to a heteroalkyl wherein one or more hydrogens of the hydrocarbon group are replaced with one or more substituents selected from the group consisting of halogen (preferably -F or -Cl), -OH, -CN, -SH, -NH₂, -NHCH₃, -NO₂, -C₁₋₂ alkyl substituted with 1 to 6 halogens (preferably -F or -Cl, more preferably -F), -CF₃, -OCH₃, -OCF₃, and -(CH₂)₀₋₄-COOH. In different
35 embodiments 1, 2, 3 or 4 substituents are present.

"Alkenyl" refers to a hydrocarbon group made up of two or more carbons where one or more carbon-carbon double bonds are present. The alkenyl hydrocarbon group may be straight-chain or contain one or more branches or cyclic groups. Protecting group hydrocarbons are preferably C₂₋₄. R alkenyl groups are preferably about 4 to about 20 carbon atoms in length, more preferably C₅₋₁₅. Reference to "longer length alkenyl" indicates at least about 4 carbon atoms.

"Substituted alkenyl" refers to an alkenyl wherein one or more hydrogens are replaced with one or more substituents selected from the group consisting of halogen (preferably -F or -Cl), -OH, -CN, -SH, -NH₂, -NHCH₃, -NO₂, -C₁₋₂ alkyl substituted with 1 to 6 halogens (preferably -F or -Cl, more preferably -F), -CF₃, -OCH₃, -OCF₃, and -(CH₂)₀₋₄-COOH. In different embodiments 1, 2, 3 or 4 substituents are present.

"Aryl" refers to an optionally substituted aromatic group with at least one ring having a conjugated pi- electron system, containing up to two conjugated or fused ring systems. Aryl includes carbocyclic aryl, heterocyclic aryl and biaryl groups. Preferably, the aryl is a 5 or 6 membered ring. Preferred atoms for a heterocyclic aryl are one or more sulfur, oxygen, and/or nitrogen. Examples of aryl include phenyl, 1-naphthyl, 2-naphthyl, indole, quinoline, 2-imidazole, and 9-antracene. Aryl substituents are selected from the group consisting of -C₁₋₄ alkyl, -C₁₋₄ alkoxy, halogen (preferably -F or -Cl), -OH, -CN, -SH, -NH₂, -NO₂, -C₁₋₂ alkyl substituted with 1 to 5 halogens (preferably -F or -Cl, more preferably -F), -CF₃, -OCF₃, and -(CH₂)₀₋₄-COOH. In different embodiments the aryl contains 0, 1, 2, 3, or 4 substituents.

"Alkylaryl" refers to an "alkyl" joined to an "aryl".

GHS Receptor Binding Assay

Assays measuring the ability of a compound to bind a GHS receptor employ a GHS receptor, a fragment of the receptor comprising a ghrelin binding site, a polypeptide comprising such a fragment, or a derivative of the polypeptide. Preferably, the assay uses the GHS receptor or a fragment thereof.

A polypeptide comprising a GHS receptor fragment that binds ghrelin can also contain one or more polypeptide regions not found in a GHS receptor. A derivative of such a polypeptide comprises a GHS receptor fragment that binds ghrelin along with one or more non-peptide components.

The GHS receptor amino acid sequence involved in ghrelin binding can be readily identified using labeled ghrelin or truncated ghrelin analogs and different receptor fragments. Different strategies can be employed to select fragments to be tested to narrow down the binding region. Examples of such strategies include testing consecutive fragments about 15 amino acids in length starting at the N-terminus, and testing longer length fragments. If longer length fragments are tested, a fragment binding ghrelin can be subdivided to further locate the ghrelin binding region. Fragments used for binding studies can be generated using recombinant nucleic acid techniques.

Binding assays can be performed using individual compounds or preparations containing different numbers of compounds. A preparation containing different numbers of compounds having the ability to bind to the GHS receptor can be divided into smaller groups of compounds that can be tested to identify the compound(s) binding to the GHS receptor. In an embodiment of the present invention, a test preparation containing at least 10 compounds is used in a binding assay.

Binding assays can be performed using recombinantly produced GHS receptor polypeptides present in different environments. Such environments include, for example, cell extracts and purified cell extracts containing the GHS receptor polypeptide expressed from recombinant nucleic acid or naturally occurring nucleic acid; and also include, for example, the use of a purified GHS receptor polypeptide produced by recombinant means or from naturally occurring nucleic acid which is introduced into a different environment.

Screening for GHS Receptor Active Compounds

Screening for GHS receptor active compounds is facilitated using a recombinantly expressed receptor. Using a recombinantly expressed GHS receptor offers several advantages such as the ability to express the receptor in a defined cell system so that a response to a compound at the GHS receptor can more readily be differentiated from responses at other receptors. For example, the GHS receptor can be expressed in a cell line such as HEK 293, COS 7, and CHO not normally expressing the receptor by an expression vector, wherein the same cell line without the expression vector can act as a control.

Screening for compounds reducing GHS receptor activity is facilitated through the use of a truncated ghrelin analog in the assay. The use of a truncated

ghrelin analog in a screening assay provides for GHS receptor activity. The effect of test compounds on such activity can be measured to identify, for example, allosteric modulators and antagonists.

GHS receptor activity can be measured using different techniques such as detecting a change in the intracellular conformation of the GHS receptor, in the G-protein coupled activities, and/or in the intracellular messengers. Preferably, GHS receptor activity is measured using techniques such as those measuring intracellular Ca^{2+} . Examples of techniques well known in the art that can be employed to measure Ca^{2+} include the use of dyes such as Fura-2 and the use of Ca^{2+} -bioluminescent sensitive reporter proteins such as aequorin. An example of a cell line employing aequorin to measure G-protein activity is HEK293/aeq17. (Button *et al.*, 1993, *Cell Calcium* 14, 663-671, and Feighner *et al.*, 1999, *Science* 284, 2184-2188.)

Chimeric receptors containing a ghrelin binding region functionally coupled to a different G-protein can also be used to measure GHS receptor activity. A chimeric GHS receptor contains an N-terminal extracellular domain; a transmembrane domain made up of transmembrane regions, extracellular loop regions, and intracellular loop regions; and an intracellular carboxy terminus. Techniques for producing chimeric receptors and measuring G-protein coupled responses are provided in, for example, International Application Number WO 97/05252, and U.S. Patent Number 5,264,565, both of which are hereby incorporated by reference herein.

Stimulation of GHS Receptor Activity

Truncated ghrelin analogs can be used to stimulate GHS receptor activity. Such stimulation can be used, for example, to study the effect of GHS receptor modulation, to study the effect of growth hormone secretion, to look for or study ghrelin antagonists, or to achieve a beneficial effect in a subject. Beneficial effects that can be achieved include one or more of the following: treating a growth hormone deficient state, increasing muscle mass, increasing bone density, treating sexual dysfunction in males or females, facilitating a weight gain, facilitating maintenance of weight, facilitating maintenance of physical functioning, facilitating recovery of physical function, and/or facilitating appetite increase.

Increasing weight or appetite can be useful for maintaining weight or producing a weight or appetite gain in an under weight subject, or in a patient having a disease or undergoing treatment that affects weight or appetite. In addition, for example, farm animals such as pigs, cows and chickens can be treated to gain weight.

Under weight subjects include those having a body weight about 10% or less, 20% or less, or 30% or less, than the lower end of a "normal" weight range or Body Mass Index ("BMI"). "Normal" weight ranges are well known in the art and take into account factors such as a patient age, height, and body type.

- 5 BMI measures your height/weight ratio. It is determined by calculating weight in kilograms divided by the square of height in meters. The BMI "normal" range is 19-22.

Administration

- 10 Truncated ghrelin analogs can be formulated and administered to a subject using the guidance provided herein along with techniques well known in the art. The preferred route of administration ensures that an effective amount of compound reaches the target. Guidelines for pharmaceutical administration in general are provided in, for example, *Remington's Pharmaceutical Sciences 18th Edition*, Ed. Gennaro, Mack Publishing, 1990, and *Modern Pharmaceutics 2nd Edition*, Eds. Banker and Rhodes, Marcel Dekker, Inc., 1990, both of which are hereby incorporated by reference herein.

- Truncated ghrelin analogs can be prepared as acidic or basic salts. Pharmaceutically acceptable salts (in the form of water- or oil-soluble or dispersible products) include conventional non-toxic salts or the quaternary ammonium salts that are formed, *e.g.*, from inorganic or organic acids or bases. Examples of such salts include acid addition salts such as acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, 25 glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate; and base salts such as ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts 30 such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine and lysine.

- Truncated ghrelin analogs can be administered using different routes including oral, nasal, by injection, transdermal, and transmucosally. Active 35

ingredients to be administered orally as a suspension can be prepared according to techniques well known in the art of pharmaceutical formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavoring agents. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants.

Administered by nasal aerosol or inhalation formulations may be prepared, for example, as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, employing fluorocarbons, and/or employing other solubilizing or dispersing agents.

Truncated ghrelin analogs may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form. When administered by injection, the injectable solution or suspension may be formulated using suitable non-toxic, parenterally-acceptable diluents or solvents, such as Ringer's solution or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

Suitable dosing regimens are preferably determined taking into account factors well known in the art including type of subject being dosed; age, weight, sex and medical condition of the subject; the route of administration; the renal and hepatic function of the subject; the desired effect; and the particular compound employed.

Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug. The daily dose for a subject is expected to be between 0.01 and 1,000 mg per subject per day.

Truncated ghrelin analogs can be provided in kit. Such a kit typically contains an active compound in dosage forms for administration. A dosage form contains a sufficient amount of active compound such that a desirable effect can be obtained when administered to a subject during regular intervals, such as 1 to 6 times a day, during the course of 1 or more days. Preferably, a kit contains instructions

indicating the use of the dosage form to achieve a desirable affect and the amount of dosage form to be taken over a specified time period.

EXAMPLES

- 5 Examples are provided below to further illustrate different features of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: Peptide Synthesis Purification and Characterization

- 10 Fmoc-protected amino acids were obtained from AnaSpec (San Jose, California), 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin from PE Biosystems (Foster City, California), Boc-7-aminoheptanoic acid from Bachem (King of Prussia, Pennsylvania) and n-octanoic acid, 2,4,6-octatrienoic acid, 2-propylpentanoic acid, 11-undecanoic acid, palmitic acid, 8-bromooctanoic acid, 1-
15 adamanteneacetic acid and benzoic acid from Aldrich (Milwaukee, Wisconsin).

- Elongation of peptidyl chains on 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin was performed on a 431A ABI peptide synthesizer. Manufacture-supplied protocols were applied for coupling of the hydroxybenzotriazole esters of amino acids in N-methylpyrrolidone (NMP). The
20 fluorenylmethyloxycarbonyl (Fmoc) group was used as a semipermanent α -amino protecting group, whereas the side chains protecting groups were: *tert*-butyl for serine, trityl for serine in position 3 and for histidine and glutamine, *tert*-butyl ester for glutamic acid, 2,2,4,6,7, -pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine, *tert*-buthyloxycarbonyl (Boc) for lysine and for α -amino group of glycine in
25 position 1.

- The peptidyl resin was then transferred into a vessel and the trityl group from the side chain of Ser³ was manually removed with 1% trifluoroacetic acid (TFA) in dichloromethane (45 minutes at room temperature). The peptidyl resin was thoroughly washed, and then agitated for 4 hours with the 6-fold excess of each 1-
30 ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) and a selected acid in DCM or NMP, in the presence of a catalytic amount of 4-dimethylaminopyridine (ca. 10 mg). The peptidyl resin was again washed with DCM, NMP and methanol, dried and treated with TFA in the presence of scavengers (ca. 3% total of the mixture of water-anisole -triethylsilane, 1:1:1, v/v/v). After 1.5 hours, the resin was filtered off, TFA

was removed *in vacuo* and the residue was triturated with ether. The precipitate which formed was filtered off, washed thoroughly with ether and dried.

The crude peptide was analyzed by analytical reverse-phase high-pressure liquid chromatography (RP HPLC) on a C18 Vydac column attached to a Waters 600E system with automatic Wisp 712 injector and 991 Photodiode Array detector. A standard gradient system of 0-100% buffer B in 30 minutes (G1), or a gradient of 20-80% buffer B in 30 minutes (G2), was used for analysis; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in acetonitrile. HPLC profiles were recorded at 210 nm and 230 nm.

Preparative separations were performed on a Waters Delta Prep 4000 system with a semipreparative C18 RP Vydac column. The above-described solvent system of water and acetonitrile, in a gradient of 20-80% buffer B in 60 minutes (G3) or in a gradient of 0-60% buffer B, was used for separations.

The chromatographically homogenous compounds were analyzed by electrospray mass spectrometry (Hewlett Packard Series 1100 MSD spectrometer).

Example 2: Binding Assay

Binding of [³⁵S]-MK-677 to crude membranes prepared from HEK 293-aequorin stable cell lines was performed as described in Chen *et al.*, *Bioorg. Med. Chem. Lett.* 1996, 6, 2163-2169, and Howard *et al.*, *Science* 1996, 273, 974-977. Prior to the binding assay, the HEK 293-AEQ17 cells (8 x 10⁵ cells plated 18 hours before transfection in a T75 flask) were transfected with 22 µg of human GHS receptor plasmid using 264 µg lipofectamine. The open reading frame cDNA (SEQ. ID. NO. 22) encoding the human GHS receptor inserted in the mammalian expression vector pcDNA-3 (Invitrogen, Carlsbad, CA) was used for binding and expression studies.

For a 96-well filter binding assay, 0.05 nM [³⁵S]-MK-677 (specific activity ~1200Ci/mmol) was bound to 4 µg membrane protein per well with or without competing test ligand. The bound membranes were filtered on 0.5% polyethylenimine prewet filters (UniFilter 96 GF/C; Packard #6005174, Meriden, CT). Filters were washed 8 times, dried, and counted with Microscint 20 (Packard #6013621, Meriden, CT). IC₅₀ values were determined from three separate assays performed in triplicate.

SEQ. ID. NO. 22 is as follows:

ATGTGGAACGCGACGCCCAGCGAAGAGCCGGGGTTCAACCTCACACTGGC
 CGACCTGGACTGGGATGCTTCCCCCGGCAACGACTCGCTGGGCGACGAGC
 TGCTGCAGCTCTTCCCCGCGCCGCTGCTGGCGGGCGTCACAGCCACCTGC
 5 GTGGCACTCTTCGTGGTGGGTATCGCTGGCAACCTGCTCACCATGCTGGTG
 GTGTCGCGCTTCCGCGAGCTGCGCACCACCACCAACCTCTACCTGTCCAG
 CATGGCCTTCTCCGATCTGCTCATCTTCCTCTGCATGCCCCTGGACCTCGTT
 CGCCTCTGGCAGTACCGGCCCTGGAACCTCGGCGACCTCCTCTGCAAACCT
 CTTCCAATTCGTCAGTGAGAGCTGCACCTACGCCACGGTGCTCACCATCAC
 10 AGCGCTGAGCGTCGAGCGCTACTTCGCCATCTGCTTCCCACTCCGGGCCA
 AGGTGGTGGTCACCAAGGGGGCGGGTGAAGCTGGTCATCTTCGTCATCTGG
 GCCGTGGCCTTCTGCAGCGCCGGGGCCCATCTTCGTGCTAGTCGGGGTGGA
 GCACGAGAACGGCACCGACCTTGGGACACCAACGAGTGCCGCCCCACC
 GAGTTTGCGGTGCGCTCTGGACTGCTCACGGTCATGGTGTGGGTGTCCAG
 15 CATCTTCTTCTTCCTTCCTGTCTTCTGTCTCACGGTCCTCTACAGTCTCATC
 GGCAGGAAGCTGTGGCGGAGGAGGCGGGCGATGCTGTCGTGGGTGCCT
 CGCTCAGGGACCAGAACCACAAGCAAACCGTGAAAATGCTGGCTGTAGT
 GGTGTTTGCCCTTCATCCTCTGCTGGCTCCCCCTCCACGTAGGGCGATATTT
 ATTTTCCAAATCCTTTGAGCCTGGCTCCTTGGAGATTGCTCAGATCAGCCA
 20 GTACTGCAACCTCGTGTCTTTGTCCTTCTTACCTCAGTGCTGCCATCAA
 CCCCATTTCTGTACAACATCATGTCCAAGAAGTACCGGGTGGCAGTGTTCA
 GACTTCTGGGATTCTGAACCTTCTCCCAGAGAAAGCTCTCCACTCTGAAA
 GATGAAAGTTCTCGGGCCTGGACAGAATCTAGTATTAATACATGA

25 Example 3: Aequorin Bioluminescence Functional Assay

The aequorin bioluminescence assay is a reliable test for identifying G-protein coupled receptors which couple through the G α protein subunit family consisting of Gq and G11. Coupling through Gq and G11 leads to the activation of phospholipase C, mobilization of intracellular calcium and activation of protein
 30 kinase C. A stable cell line expressing the human GHSR1a and the aequorin reporter protein were used for the assay. (Button *et al.*, *Cell Calcium* 1993, 14, 663-671.)

The assay was performed using a Luminoskan RT luminometer (Labsystems Inc., Gaithersburg, MD) controlled by custom software written for a Macintosh PowerPC 6100. 293AEQ17/GHSR1a cells (as described in Example 2),
 35 were cultured for 72 hours and the apo-aequorin in the cells was charged for 1 hour

with coelenterazine (10 μ M) under reducing conditions (300 μ M reduced glutathione) in ECB buffer (140 mM NaCl, 20 mM KCl, 20 mM HEPES-NaOH [pH=7.4], 5 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mg/ml bovine serum albumin). The cells were harvested, washed once in ECB medium and resuspended to 500,000 cells/ml. 100 ml of cell suspension (corresponding to 5×10^4 cells) was then injected into the test plate containing the peptides, and the integrated light emission was recorded over 30 seconds, in 0.5 second units. 20 μ L of lysis buffer (0.1% final Triton X-100 concentration) was then injected and the integrated light emission recorded over 10 seconds, in 0.5 second units. The "fractional response" values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response. The functional EC₅₀ values were measured in three separate assays.

Example 4: Activity of Different Types of Ghrelin Analogs

Analogs of human ghrelin (Tables 1-4) were prepared by solid phase syntheses as described in Example 1. The analogs were evaluated for their binding affinities to the cloned human GHS receptor 1a in a competitive binding assay with [³⁵S]-MK-677 as the radiolabeled ligand, and also for their ability to stimulate inositol triphosphate-coupled mobilization of intracellular calcium in HEK-293 cells expressing hGHSR1a, as described in Examples 2 and 3.

The role of the n-octanoyl group in interaction of human ghrelin with hGHSR1a was examined by testing compounds in which the hydroxyl group in the side chain of Ser³ was acylated by various aliphatic or aromatic acids (see Table 1). Acylation of Ser³ with hydrophobic acids resembling in size n-octanoic acid, such as the unsaturated 2,4,6-octatrienoic acid or the branched 2-propylpentanoic acid, or the longer chain 11-undecanoic acid or palmitic acid, yielded compounds 1-4 having agonist potencies similar to that of ghrelin. In contrast, replacement of the n-octanoyl group with the substantially smaller acetyl group led to compound 5 which was 20-fold less potent than human ghrelin in the hGHSR1a activation assay. Ghrelin without the n-octanoyl group (des-octanoyl-ghrelin) poorly activated hGHSR1a even at micromolar concentrations (compound 6 in Table 1).

To explore possible non-hydrophobic interactions with the receptor, the hydroxyl group of serine 3 in compounds 7, 8, 9 was acylated with aliphatic acids which mimic the extended hydrophobic chain of n-octanoic acid, but also contain

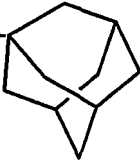
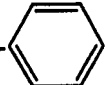
bromo, amino or amido groups. In the hGHSR1a activation assay, analogs with amido and amino groups in the side chain of residue 3 were respectively 5- and 20-fold less potent as agonists at hGHSR1a (compounds 8 and 9 in Table 1), whereas, compound 7 with 8-bromooctanoyl group attached to the side chain of Ser³ retained the potency of the parent compound. Compound 10 having the bulky and rigid hydrophobic 1-adamantaneacetyl group in place of n-octanoyl group in position 3 activated hGHSR1a as efficiently as ghrelin, while compound 11 having a smaller benzoyl group in the same position was 2-fold less potent.

Table 2 illustrates the effect of attaching the n-octanoyl group to the side chain of residue 3 through an amide bond. In compounds 12 and 14, 2,3-di-amino-propionic acid was incorporated in place of Ser³ and the β -amino group of this new residue was acylated with n-octanoic acid. Compounds of 12 and 14 activated hGHSR1a as efficiently as the parent compounds, ghrelin and the compound 13 (peptide encompassing 1 to 14 residues of ghrelin).

Table 3 provides binding and functional data on ghrelin analogs with the hydroxyl groups of serine residues other than Ser³ esterified by n-octanoic acid. Compound 15 with modified Ser², was active. Compounds 16 and 17, with modified Ser⁶ or Ser¹⁸, were inactive even at micromolar concentrations.

Table 4 provides binding and functional data on truncated analogs. Shortening of ghrelin down to its 4 amino terminus residues yielded active peptides. The relative binding affinities gradually decreased with the extent of the C-terminal deletions. Short peptides encompassing residue 1-3, or 2-5, or 3-5, or 2-4 of ghrelin were poor activators even at micromolar concentrations.

Table 1
Analogues of Human Ghrelin Modified at Position 3

Compound	X	Binding Assay	Functional Assay	
		IC ₅₀ (nM)	EC ₅₀ (nM)	% activation at 10 μM relative to ghrelin
Human Ghrelin	CO-(CH ₂) ₆ CH ₃	0.25 ± 0.07	32 ± 4.5	100
1	CO-CH=CH-CH=CH-CH=CH-CH ₃	0.98 ± 0.36	39 ± 10	108 ± 1
2	CO-CH(CH ₂ CH ₂ CH ₃) ₂	0.96 ± 0.05	38 ± 11	103 ± 1
3	CO-(CH ₂) ₉ CH ₃	0.12 ± 0.03	9.1 ± 6.2	104 ± 3
4	CO-(CH ₂) ₁₄ CH ₃	0.87 ± 0.17	8.3 ± 0.6	96 ± 11
5	CO-CH ₃	> 2000	2000 ± 480	59 ± 13
6	-----	> 10,000	>10,000	41 ± 4
7	CO-(CH ₂) ₆ CH ₂ Br	0.08 ± 0.0	18 ± 0.9	88 ± 7
8	CO-(CH ₂) ₂ CO-NH-(CH ₂) ₂ CH ₃	1020 ± 202	410 ± 120	86 ± 10
9	CO-(CH ₂) ₆ NH ₂	> 2000	1200 ± 370	68 ± 3
10	CO-CH ₂ 	0.12 ± 0.05	24 ± 9.5	95 ± 7
11	CO- 	11 ± 1.5	53 ± 3.2	85 ± 2

The modification shown in Table 1 is with respect to:

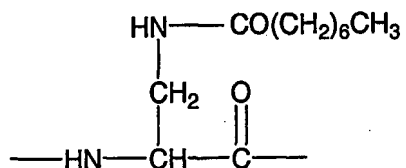


Compounds 1-11 correspond to SEQ. ID. NOs. 7-17.

Table 2
Analogues of Human Ghrelin with an Amide Bond in the Side Chain of Residue 3

Compound	Binding Assay IC ₅₀ (nM)	Functional Assay	
		EC ₅₀ (nM)	% activation at 10 µM relative to ghrelin
Human Ghrelin	0.25 ± 0.07	32 ± 4.5	100
12	0.42 ± 12	31 ± 9.8	105 ± 5
13	9.6 ± 1.5	17 ± 4	97 ± 9
14	8 ± 2.7	38 ± 1.8	102 ± 3

- 5 Compound 12 is a modified amino acid having the following sequence GSX^bFLSPEHQRVQQRKESKKPPAKLQPR (SEQ. ID. NO. 18), where X^b is



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Table 3
Analogues of Human Ghrelin Octanoylated at Other Serine Residues

Compound	Binding Assay IC ₅₀ (nM)	Functional Assay	
		EC ₅₀ (nM)	% activation at 10 µM relative to ghrelin
Human Ghrelin	0.25 ± 0.07	32 ± 4.5	100
15	48 ± 7.2	42 ± 14	81 ± 14
16	> 1000	> 10,000	36 ± 1
17	> 5000	> 10,000	46 ± 2

- 15 Compounds 15-17 are as follows:

GXSFLSPEHQRVQQRKESKKPPAKLQPR (compound 15, SEQ. ID. NO. 19),
 GSSFLXPEHQRVQQRKESKKPPAKLQPR (compound 16, SEQ. ID. NO. 20),
 GSSFLSPEHQRVQQRKEXKKPPAKLQPR (compound 17, SEQ. ID. NO. 21),
 wherein X is

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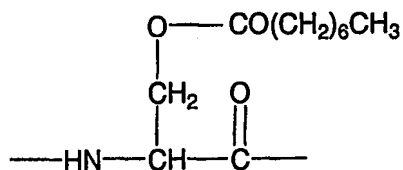


Table 4
 Truncate Analogs of Human Ghrelin

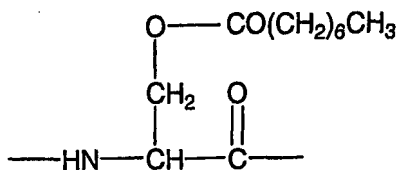
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Compound	Binding Assay	Functional Assay	
	IC ₅₀ (nM)	EC ₅₀ (nM)	% activation at 10 μ M relative to ghrelin
Human Ghrelin	0.25 \pm 0.07	32 \pm 4.5	100
18	0.16 \pm 0.02	15 \pm 4.5	100 \pm 4
19	0.77 \pm 0.18	22 \pm 16	92 \pm 16
13	9.6 \pm 1.5	17 \pm 4	97 \pm 9
20	7.1 \pm 5.7	20 \pm 6	89 \pm 17
21	55 \pm 10	11.5 \pm 2.3	96 \pm 7
22	889 \pm 72	72 \pm 29	91 \pm 4
23	> 2000	1150 \pm 120	30 \pm 5
24	> 10,000	>10,000	28 \pm 1
25	> 10,000	2500 \pm 1200	29 \pm 7
26	> 10,000	>10,000	28 \pm 1

Compounds 23-26 are as follows:

- 15 Ac- SXFL-NH₂ (compound 23),
 GSX-NH₂ (compound 24),
 Ac- XFL-NH₂ (compound 25),
 Ac- SXF-NH₂ (compound 26),

where X is



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Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. A truncated ghrelin analog having a structure selected from the group consisting of:

- a) Z^1 -GSXF(Z)_n-Z²; and
 b) Z^1 -GXSF(Z)_n-Z²;

wherein X is a modified amino acid containing a bulky hydrophobic R group;

each Z is independently either alanine, valine, leucine, isoleucine, proline, tryptophan, phenylalanine, methionine, glycine, serine, threonine, tyrosine, cysteine, asparagine, glutamine, lysine, arginine, histidine, aspartic acid, or glutamic acid, or a derivative thereof;

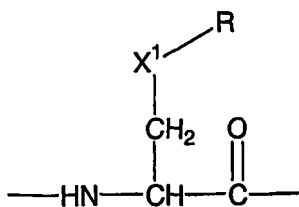
Z¹ is an optionally present protecting group that, if present, is covalently joined to the N-terminal amino group;

Z² is an optionally present protecting group that, if present, is covalently joined to the C-terminal carboxy group; and

n is 0 to 19;

or a pharmaceutically acceptable salt thereof.

2. The analog of claim 1, wherein X has the structure:



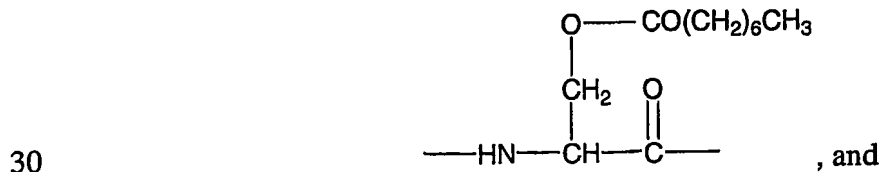
wherein X¹ is either -O-, -S-, -OC(O)-, -NHC(O)-, or -CH₂-; and

R is either -C₄₋₂₀ alkyl, -C₄₋₂₀ substituted alkyl, -C₄₋₂₀ substituted alkenyl, -C₄₋₂₀ alkenyl, -C₄₋₂₀ heteroalkyl, -C₄₋₂₀ substituted heteroalkyl, aryl, or alkylaryl.

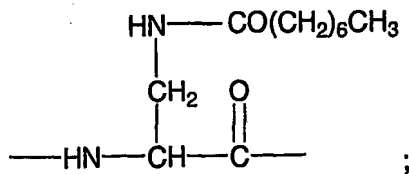
3. The analog of claim 2, wherein said analog has the structure:
 Z^1 -GSXF(Z)_n-Z².

4. The analog of claim 3, wherein n is 0-11.
5. The analog of claim 4, wherein n is 0-6.
- 5 6. The analog of claim 5, wherein n is 0-3.
7. The analog of claim 6, wherein n is 0.
- 10 8. The analog of any one of claims 1-7, wherein X¹ is -C(O)- or -NH(O)-; and R is a -C₅₋₁₅ alkyl.
9. The analog of claim 8, wherein X¹ is -C(O)- and R is -(CH₂)₆CH₃.
- 15 10. The analog of claim 9, wherein Z¹ if present is -C(O)CH₃ and Z² if present is -NH₂.
11. The analog of claim 1, wherein said analog has a modified amino acid sequence selected from the group consisting of:
 20 GSXFLSPEHQRVQQRKESKKPPA- NH₂ (SEQ. ID. NO. 4),
 GSXFLSPEHQRVQQRKES- NH₂ (SEQ. ID. NO. 5),
 GSXFLSPEHQRVQQ (SEQ. ID. NO. 2),
 GSX^bFLSPEHQRVQQ (SEQ. ID. NO. 3),
 25 GSXFLSPEHQ-NH₂ (SEQ. ID. NO. 6),
 GSXFL-NH₂ and
 GSXF-NH₂;

provided that X is



X^b is



or a pharmaceutically acceptable salt thereof.

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15. A method for achieving a beneficial effect in a subject comprising the step of administering to the subject an effective amount of the analog of claim 1.

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16. The method of claim 15, wherein said analog is selected from the group consisting of:

GSXFLSPEHQRVQQRKESKKPPA- NH_2 (SEQ. ID. NO. 4),

GSXFLSPEHQRVQQRKES- NH_2 (SEQ. ID. NO. 5),

GSXFLSPEHQRVQQ (SEQ. ID. NO. 2),

15 GSX^bFLSPEHQRVQQ (SEQ. ID. NO. 3),

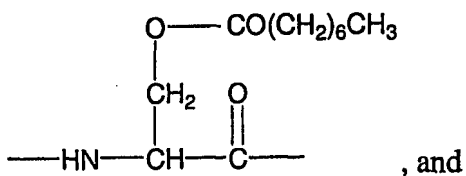
GSXFLSPEHQ- NH_2 (SEQ. ID. NO. 6),

GSXFL- NH_2 and

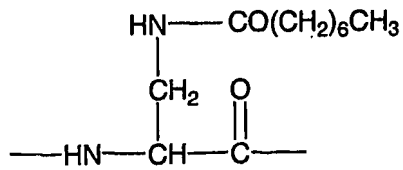
GSXF- NH_2 ;

provided that X is

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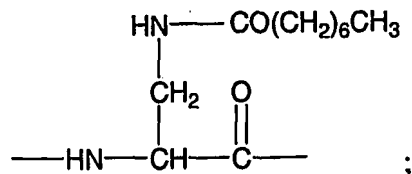
X^b is



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or a pharmaceutically acceptable salt thereof.

X^b is



or a pharmaceutically acceptable salt thereof.

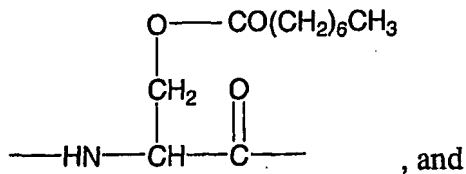
- 5 12. A method of screening for a compound able to bind to a growth-hormone secretagogue receptor comprising the step of measuring the ability of said compound to effect binding of the analog of claim 1 to either said receptor, a fragment of said receptor comprising a ghrelin binding site, a polypeptide comprising said fragment, or a derivative of said polypeptide.

10

13. The method of claim 12, wherein said method measures the ability of said analog to bind to said receptor.

14. The method of claim 13, wherein said analog is selected from
15 the group consisting of:
GSXFLSPEHQRVQQRKESKKPPA-NH₂ (SEQ. ID. NO. 4),
GSXFLSPEHQRVQQRKES-NH₂ (SEQ. ID. NO. 5),
GSXFLSPEHQRVQQ (SEQ. ID. NO. 2),
GSX^bFLSPEHQRVQQ (SEQ. ID. NO. 3),
20 GSXFLSPEHQ-NH₂ (SEQ. ID. NO. 6),
GSXFL-NH₂ and
GSXF-NH₂;

provided that X is



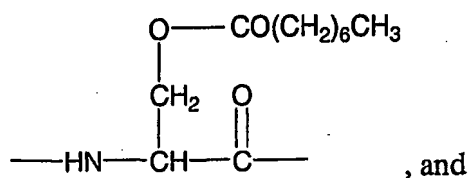
25

17. A method for stimulating growth hormone secretion comprising the step of administering to a subject an effective amount of the analog of claim 1.

- 5 18. The method of claim 17, wherein said analog is selected from the group consisting of:
 GSXFLSPEHQRVQQRKESKKPPA- NH₂ (SEQ. ID. NO. 4),
 GSXFLSPEHQRVQQRKES- NH₂ (SEQ. ID. NO. 5),
 GSXFLSPEHQRVQQ (SEQ. ID. NO. 2),
 10 GSX^bFLSPEHQRVQQ (SEQ. ID. NO. 3),
 GSXFLSPEHQ-NH₂ (SEQ. ID. NO. 6),
 GSXFL-NH₂ and
 GSXF-NH₂;

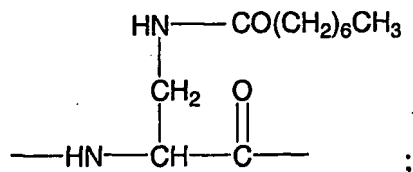
provided that X is

15



X^b is

20



or a pharmaceutically acceptable salt thereof.

SEQUENCE LISTING

<110> Merck & Co., Inc.

<120> GHRELIN ANALOGS

<130> 20661 PCT

<150> 60/207,920

<151> 2000-05-30

<160> 22

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 28

<212> PRT

<213> Human

<220>

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<400> 1'

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Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 2

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(CO-(CH₂)₆-CH₃)

<400> 2

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1				5					10				

<210> 3

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Dpr(CO-(CH₂)₆-CH₃)

<400> 3

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1				5					10				

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 <211> 23
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 <213> Artificial Sequence

<220>
 <223> Ghrelin analog

<221> AMIDATION
 <222> (23)...(23)

<221> MOD_RES
 <222> (3)...(3)
 <223> Xaa = Ser(CO-(CH₂)₆-CH₃)

<400> 4
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 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala
 20

<210> 5
 <211> 18
 <212> PRT
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<220>
 <223> Ghrelin analog

<221> AMIDATION
 <222> (18)...(18)

<221> MOD_RES
 <222> (3)...(3)
 <223> Xaa = Ser(CO-(CH₂)₆-CH₃)

<400> 5
 Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser

<210> 6
 <211> 10
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<220>
 <223> Ghrelin analog

<221> AMIDATION
 <222> (10)...(10)

<221> MOD_RES
 <222> (3)...(3)
 <223> Xaa = Ser(CO-(CH₂)₆-CH₃)

<400> 6
 Gly Ser Xaa Phe Leu Ser Pro Glu His Gln
 1 5 10

<210> 7

<211> 28
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 <213> Artificial Sequence

<220>
 <223> Ghrelin analog

<221> MOD_RES
 <222> (3)...(3)
 <223> Xaa = Ser(CO-CH=CH-CH=CH-CH=CH-CH3)

<400> 7
 Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 8
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<221> MOD_RES
 <222> (3)...(3)
 <223> Xaa = Ser(CO-CH(CH2CH2CH3)2)

<400> 8
 Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 9
 <211> 28
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<220>
 <223> Ghrelin analog

<221> MOD_RES
 <222> (3)...(3)
 <223> Xaa = Ser(CO-(CH2)9-CH3)

<400> 9
 Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 10
 <211> 28
 <212> PRT
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<220>
 <223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(CO-(CH₂)₁₄-CH₃)

<400> 10

Gly	Ser	Xaa	Phe	Leu	Ser	Pro	Glu	His	Gln	Arg	Val	Gln	Gln	Arg	Lys
1				5					10					15	
Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 11

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(CO-CH₃)

<400> 11

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1				5					10					15	
Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 12

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<400> 12

Gly	Ser	Ser	Phe	Leu	Ser	Pro	Glu	His	Gln	Arg	Val	Gln	Gln	Arg	Lys
1				5					10					15	
Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 13

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(CO-(CH₂)₆-CH₂Br)

<400> 13

Gly	Ser	Xaa	Phe	Leu	Ser	Pro	Glu	His	Gln	Arg	Val	Gln	Gln	Arg	Lys
1				5					10					15	
Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 14

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(CO-(CH₂)₂-CO-NH-(CH₂)₂-CH₃)

<400> 14

Gly	Ser	Xaa	Phe	Leu	Ser	Pro	Glu	His	Gln	Arg	Val	Gln	Gln	Arg	Lys
1				5					10					15	
Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 15

<211> 28

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<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(CO-(CH₂)₆-NH₂)

<400> 15

Gly	Ser	Xaa	Phe	Leu	Ser	Pro	Glu	His	Gln	Arg	Val	Gln	Gln	Arg	Lys
1				5					10					15	
Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 16

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(1-adamantaneacetyl)

<400> 16

Gly	Ser	Xaa	Phe	Leu	Ser	Pro	Glu	His	Gln	Arg	Val	Gln	Gln	Arg	Lys
1				5					10					15	
Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg				
			20					25							

<210> 17

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Ghrelin analog

<221> MOD_RES

<222> (3)...(3)

<223> Xaa = Ser(CO-C₆H₆)

<400> 17
 Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 18
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 <223> Ghrelin analog

<221> MOD_RES
 <222> (3)...(3)
 <223> Xaa = Dpr(CO-(CH₂)₆-CH₃)

<400> 18
 Gly Ser Xaa Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 19
 <211> 28
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<220>
 <223> Ghrelin analog

<221> MOD_RES
 <222> (2)...(2)
 <223> Xaa = Ser(CO-(CH₂)₆-CH₃)

<400> 19
 Gly Xaa Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 20
 <211> 28
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<220>
 <223> Ghrelin analog

<221> MOD_RES
 <222> (6)...(6)
 <223> Xaa = Ser(CO-(CH₂)₆-CH₃)

<400> 20
 Gly Ser Ser Phe Leu Xaa Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 21

<211> 28
 <212> PRT
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<220>
 <223> Ghrelin analog

<221> MOD_RES
 <222> (18)...(18)
 <223> Xaa = Ser(CO-(CH₂)₆-CH₃)

<400> 21
 Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys
 1 5 10 15
 Glu Xaa Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg
 20 25

<210> 22
 <211> 1101
 <212> DNA
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<220>
 <223> Human GHS receptor cDNA

<400> 22
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 ccgctgctgg cgggcgtcac agccacctgc gtggcactct tcgtgggtggg tatcgctggc 180
 aacctgctca ccatgctggt ggtgtcgcgc ttccgcgagc tgcgcaccac caccaacctc 240
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 cgctacttcg ccatctgctt cccactccgg gccaaagggtg tggtcaccaa ggggcgggtg 480
 aagctgggtca tcttcgtcat ctgggcccgtg gccttctgca gcgccgggccc catcttcgtg 540
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 gaatctagta ttaatacatg a 1101

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
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WO 01/092292 A3

(51) International Patent Classification⁷: **A61K 38/00**,
38/04, 38/24, 38/27

[US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

(21) International Application Number: PCT/US01/17026

(74) Common Representative: **MERCK & CO., INC.**; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

(22) International Filing Date: 25 May 2001 (25.05.2001)

(81) Designated States (*national*): CA, JP, US.

(25) Filing Language: English

(84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

(26) Publication Language: English

(30) Priority Data:
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Published:
— with international search report

(71) Applicant (*for all designated States except US*): **MERCK & CO., INC.** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

(88) Date of publication of the international search report:
14 August 2003

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **BEDNAREK, Maria**

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: GHRELIN ANALOGS

(57) Abstract: The present invention features truncated ghrelin analogs active at the GHS receptor. Ghrelin is a naturally occurring modified peptide. The analogs can bind to the GHS receptor and, preferably, bring about signal transduction. Ghrelin analogs have a variety of different uses including being used as a research tool and being used therapeutically.



WO 01/092292 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/17026

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00, 38/04, 38/24, 38/27

US CL : 514/2, 12, 13, 14, 15; 530/399, 328, 327, 326, 324

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 13, 14, 15; 530/399, 328, 327, 326, 324; 435/7.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOJIMA et al. Ghrelin is a Growth-Hormone-Releasing Acylated Peptide from Stomach, Nature. 09 December 1999, Vol. 402, pages 656-660, especially page 657-658.	1-14
A	KINEMAN et al. Growth Hormone (GH)-Releasing Hormone (GHRH) and the GH Secretagogue (GHS), L692,585, Differentially Modulate Rat Pituitary GHS Receptor and GHRH Receptor Messenger Ribonucleic Acid Levels. Endocrinology. 1999, Vol. 140, No. 8, pages 3581-3586.	
A	HOWARD et al. A Receptor in Pituitary and Hypothalamus that Functions in Growth Hormone Release. Science. 16 August 1996, Vol. 273, pages 974-977.	
A	CHEN, C. Growth Hormone Secretagogue Actions on the Pituitary Gland: Multiple Receptors for Multiple Ligands? Clin. Exp. Pharm. Phys. 2000, Vol. 27, pages 323-329.	

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:		"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 October 2002 (17.10.2002)

Date of mailing of the international search report

11 FEB 2003

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No.

Authorized officer

Billy Dell Chism

Telephone No. 7033080196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/17026

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-14

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-14, drawn to ghrelin analog and method of using ghrelin analog for screening for compounds effective on the binding of ghrelin analog to ghrelin binding site.

Group II, claim(s) 15-18, drawn to method of using ghrelin analog for treatment of a subject.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking Groups I-II appears to be that they all relate to a method of using the ghrelin analog.

However, the methods of using the ghrelin analog do not require the steps. The method of using the ghrelin analog in Group I requires the use of the ghrelin analog in a series of screening steps to elucidate a compound to effect binding activity of the analog to a receptor site. Consequently, the method steps of Group I are not required in the method steps of Group II wherein the ghrelin analog is used for treatment of a subject wherein the binding activity of the analog is the desired result.

Unity of invention is required under 37 CFR 1.475, wherein it states in 37 CFR 1.475(b) that an application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to combinations specified under 37 CFR 1.475(b)(1-5). In the present application, applicant is claiming a product and two methods of using the product wherein 37 CFR 1.475(b)(2) states that an invention may only comprise a product and a process of use of said product. US Patent Number: 5,948,628 teaches the method steps of Group I wherein compounds are screened for their effectiveness on binding of a protein or analog to a receptor. Thus, the method steps of Group I are not a contribution over the art and there is no unity of invention between the invention of Groups I-II.

Accordingly, Groups I-II are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

Continuation of B. FIELDS SEARCHED Item 3:

STN, BIOSIS, MEDLINE, SCISEARCH, CAPLUS, WEST

search terms: ghrelin, analog(s), truncated, growth hormone, secretagogue(s), GHS receptor